A Thesis for the Degree of Master of Philosophy in Pharmacology

Neuroprotection of active principles from *Cudrania tricuspidata* by the induction of autophagy with mTOR inhibition in rotenone-induced Parkinson's disease model

Rotenone으로 유도한 Parkinson's disease model에서 mTOR 억제로 유도된 autophagy에 의한 꾸지뽕나무 유래 유효성분물질의 신경 보호 효과

August, 2018

Natural Products Science Major, College of Pharmacy

Master Course in the Graduate School

Seoul National University

Rangjoo Lee

Abstract

Neuroprotection of active principles from *Cudrania tricuspidata* by the induction of autophagy with mTOR inhibition in rotenone-induced Parkinson's disease model

Rangjoo Lee

Natural Products Science Major

College of Pharmacy

The Graduate School

Seoul National University

Parkinson's disease (PD) is characterized by severe motor deficits, cogwheel rigidity, bradykinesia, and the loss of dopaminergic neurons. The etiology of PD

has not been clearly identified; However, the final common pathway in PD pathogenesis leads to the excessive deposition of toxins and to misfolding of proteins such as α-synuclein, and failure to degrade impaired protein might lead to the neuronal cell death associated with PD. Autophagy is a self-degradative process that removes aggregated proteins, damaged organelles, and intracellular pathogens. Recent studies have demonstrated dysregulation of the autophagy pathway in the brains of PD patients and in animal models of PD, suggesting a pivotal role for autophagy in the pathogenesis of PD. Rotenone, a common pesticide and inhibitor of mitochondrial complex I, induces loss of dopaminergic neurons and consequential aspects of PD. In this study, the effects of natural products on rotenone-mediated signaling in SH-SY5Y neuroblastoma cell were investigated to discover new lead compounds for the treatment of PD.

Cudrania tricuspidata (Moraceae) is a subtropical tree that is widely distributed in Korea, China, and Japan. The fruits of *C. tricuspidata* are used in jams, juices, and a fermented alcoholic beverage with sugar, and they are commercially produced as food in Korea. Also, the cortex and rood bark of *C. tricuspidata* have been used as a traditional medicine for inflammation and tumors. A recent study demonstrated that the extracts of *C. tricuspidata* protect neurons against oxidative stress-induced cytotoxicity and have inhibitory effects on nitric oxide synthase (NOS). The compounds isolated from *C. tricuspidata* are primarily xanthones and flavones in addition to some alkaloids, lignins, coumarins, polysaccharides, and

chromones. The xanthones from the root bark of *C. tricuspidata* have been reported to exert protective effects against 6-OHDA-induced neurotoxicity.

In this study, the effects of active compounds from the *C. tricuspidata* extracts on inducing autophagy were studied. Gerontoxanthone C (GXC) isolated from the root barks of the *C. tricuspidata* protected against rotenone-induced neuronal cell death and the collapse of mitochondrial membrane potential (MMP) through induction autophagy with mTOR inhibition in SH-SY5Y cells. Based on the results, it was suggested that GXC might be promising candidates for the therapy of familiar PD via facilitating recovery of damaged organelles.

Key words: Parkinson's disease, PD, neuroprotection, autophagy, mTOR, *Cudrania tricuspidata*

***Student Number* : 2016-29887**

Table of Contents

List of Figures

List of Tables

Neuroprotection of active principles from *Cudrania tricuspidata* by the induction of autophagy with mTOR inhibition in rotenone-induced Parkinson's disease model

1. Introduction

Parkinson's disease (PD) is a common progressive neurodegenerative disorder affecting more than 6 million people worldwide. The disease is characterized clinically by a triad of cardinal motor symptoms including bradykinesia, tremors and rigidity, and pathologically by the selective loss of dopamine neurons in the substantia nigra pars compacta (SNpc) and the formation of Lewy bodies. Although the etiology of PD is still unclear, there is growing evidence indicating that intracellularly oxidative stress, mitochondrial damage, lysosomal dysfunction, neuro inflammatory changes and formation of pathologic inclusions contribute to the pathology of the disease. Treatment of PD is generally symptomatic, where levodopa remains the most effective agent. However, with disease progression, levodopa medication becomes increasingly inadequate for the management of motor fluctuations and dyskinesias. Levodopa given clinically undergoes auto-oxidation and forms reactive oxygen species (ROS) which could be toxic to remaining dopamine neurons. Dopamine agonists and monoamine oxidase B inhibitors are also prescribed for the treatment of early PD as in addition to their symptomatic benefits they postpone the onset of levodopa therapy.

Autophagy is a self-degradative process that removes aggregated proteins,

damaged organelles, and intracellular pathogens. Recent studies have demonstrated dysregulation of the autophagy pathway in the brains of PD patients and in animal models of PD, suggesting a pivotal role for autophagy in the pathogenesis of PD. Furthermore, there have also been several studies showing that autophagy enhancing leads to decreased levels of toxic aggregates and could show neuroprotective effects in PD models.

Rotenone, a potent mitochondrial complex I inhibitor, is one of the most relevant neurotoxins to induce parkinsonian symptoms. Despite debates, the rotenone model is able to recapitulate slow and specific loss of dopaminergic neurons and over-expression of alpha-synuclein and better mimics the clinical features of idiopathic PD. Among the various models for PD, the rotenone model has recently drawn particular attention for two reasons: 1) it reproduces most of the motor symptoms and the histopathological features of PD, including Lewy bodies; and 2) rotenone and other pesticides are powerful inhibitors of mitochondrial respiration and associated with the higher incidence of sporadic Parkinsonism among the population of rural areas. Thus, rotenone-induced parkinsonian models were chosen to explore the role of autophagy in PD in this study.

Cudrania tricuspidata, a small thorny tree belonging to the family

Moraceae, is mainly distributed in East Asia, and its roots have long been used to treat contusion, hemoptysis, hematemesis, lumbago, and spermatorrhea. Phytochemical studies of *C. tricuspidata* have revealed that the major constituents of this plant are various types of xanthones and flavonoids, and these constituents have shown cytotoxic, antioxidant, antiatherosclerotic, anti-inflammatory, and hepato-protective activities. In a search to discover neuroprotective metabolites from natural products, a MeOH extract of *C. tricuspidata* showed significant protective effects against 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in human neuroblastoma SH-SY5Y cells. 6-OHDA, as a neurotoxin, elicits dopaminergic neuronal cell death in modeling PD.

Nevertheless, to the best of our knowledge, there has been no experiment showing the neuroprotective effect of xanthones through enhancing the autophagy pathway in a PD model. Therefore, we hypothesized that xanthones may decrease rotenone-induced neurotoxicity in SH-SY5Y cells as an in vitro model of PD by modulating the autophagy signaling pathway. In the current study, we investigated the concentration-dependent effect of GXC on autophagy markers in SH-SY5Y cells and evaluated its neuroprotective effect. Furthermore, we evaluated which levels of the autophagy signaling pathway are involved in xanthones-induced autophagy

modulation.

2. Material and methods

2.1. Chemicals and reagents

Rotenone, JC-1, and CCK-8 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Rockville, MD, USA). Hybond-polyvinylidene difluoride (PVDF) membranes were purchased from Amersham Pharmacia Biotechnology Inc. (Piscataway, NJ, USA). PRO-PREP protein extraction solution and WEST-Queen® ECL solution were purchased from iNtRON Biotech Inc. (Kyunggi, Korea). Beta-actin, LC3-beta, p62, mTOR, p-mTOR, ULK1, p-ULK1, LAMP1, LAMP2 first antibody, secondary antibody and FITC-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)

2.2. Preparation of GXC

Cudrania tricuspidata was stored at the Korea Forest Research Institute at Southern Forest Research Center (Jinju, Korea) in September 2008. A

voucher specimen (accession number KH1-4-090814) was kept at the Department of Biosystems and Biotechnology at Korea University (Seoul, Korea). GXC was isolated from the roots of *Cudrania tricuspidata* and the structure of GXC was determined by spectroscopic methods, and the purity was more than 98% (Wei and Yu, 2008). GXC was dissolved in DMSO and diluted with PBS to obtain the proper concentration. Final concentration of DMSO was less than 0.1% and it didn't influence the performed assays.

2.3. Cell cultures

The human neuroblastoma cell line SH-SY5Y (ATCC No. CRL-2266) was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10 % heat-inactivated FBS and 1 % penicillin/streptomycin at 37 ºC in a humidified 5 % CO_2 atmosphere.

2.4. Measurement of cell viability

SH-SY5Y cells were seeded at a density of 1×10^5 cells/200 μL/well in 96-well plates for 24 h, and the cells were treated with isolated compounds

from *C. tricuspidata* and rotenone for 24h or 48 h. Cell viability was determined using a CCK-8 and measured by ELISA (Koo et al., 2011).

2.5. Measurement of MMP by JC-1 staining

SH-SY5Y cells were seeded at a density of 2×10^5 cells/2 mL/well in 6-well plates for 24 h, and the cells were pre-treated with isolated compounds for 24 h followed by subsequent treatment with rotenone (0.5 μM) for an additional 2 h. MMP was determined using a JC-1 dyes. Briefly, the cells were washed with PBS, and then incubated with 10 μg/mL of JC-1 for 30 min at 37 ºC in the dark. The cells were then washed with PBS. The fluorescence intensities were measured by flow cytometry (BD FACSCalibur ^{TM}).

2.6. Measurement of protein expression

The cells were collected, washed with PBS and lysed with a PRO-PREP protein extraction solution at -20 ºC for 20 min. After centrifugation at 13,000 x g for 30 min, the supernatant was used as the total protein extracts. Western blot analysis was accomplished as previously described

method (Ham et al., 2013).

2.7. Measurement of autophagy by fluorescence microscope

The culture dish was coated with 0.2 % gelatin at 37 °C for 30 min and dried at RT on a clean bench. The SH-SY5Y cells were plated at a density of 5×10^4 cells/200 μL/well in coated dishes and treated with isolated compounds from *C. tricuspidata* and rotenone for 24 h. After treatment, the cells were washed with 1x PBS/Tween-20 buffer (pH 7.4) (PBST) once. The cells were fixed with 4 % paraformaldehyde for 30 min at room temperature (RT). After washing with PBST, the blocking steps were performed with 1 % BSA in PBST. Next, the cells were incubated with the primary antibody at 4 °C overnight. The next day, the cells were washed with PBST 3 times and incubated with FITC-conjugated secondary antibody for 1 h at RT. After 1 h, the cells were washed with PBST 3 times, and DAPI staining was performed for 5 min at RT. Lastly, the PBST washing and mounting steps were conducted.

2.8. Statistical analysis

All experimental data are expressed as mean value ± standard deviation. Statistical significance between multiple groups was determined by one-way ANOVA (PRISM Graph Pad, San Diego, CA, USA). When ANOVA had a significant difference, *post hoc* Bonferroni's multiple comparison tests was conducted. *P* value less than 0.05 was regarded to be statistically significant.

3. Results

3.1. Protective effect of GXC on rotenone-induced cytotoxicity

The isolated compounds from *C. tricuspidata* were evaluated to determine its protective effects on rotenone-induced cell death. As shown in Table 1, five isolated compounds protected rotenone-induced cell death. Among three isolated compounds, GXC showed potent neuroprotective effects on rotenone-induced cytotoxicity with an EC_{50} values of 5.6 μM. GXC protect rotenone-induced cell death concentration-dependently manner (Fig. 2).

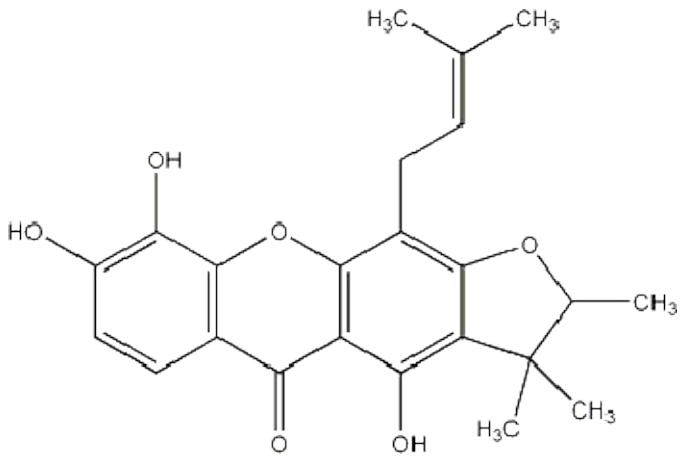

Figure 1. Chemical structure of GXC

Table 1. Protective effect of isolated compounds from *C.tricuspidata* on rotenone-induced cytotoxicity

	Cell viability EC_{50} value(μM)			Cell viability EC_{50} value(μM)	
No.	**Code**	**ROT EC_{50}**	**No.**	**Code**	**ROT EC_{50}**
1	TH1-116-2K (TH1)	>50	22	TH3-165-4K (TH22)	>50
2	TH1-116-3K (TH2)	>50	23	TH3-185-1 (TH23)	>50
3	TH3-23-5 (TH3)	>50	24	TH4-3-3 (TH24)	>50
4	TH3-33-2K2 (TH4)	>50	25	TH4-5-2 (TH25)	>50
5	TH3-33-3K1 (TH5)	>50	26	TH4-7-2K2 (TH26)	>50
6	TH3-33-4 (TH6)	>50	27	TH4-103-1 (TH27)	>50
7	TH3-33-5K1 (TH7)	>50	28	TH4-105-4K (TH28)	>50
8	TH3-77-4 (TH8)	>50	29	TH4-105-5 (TH29)	>50
9	TH3-83-2 (TH9)	>50	30	TH4-105-7K (TH30)	>50
10	TH3-83-5 (TH10)	>50	31	TH4-109-1 (TH31)	>50
11	TH3-83-7 (TH11)	>50	32	TH4-109-4 (TH32)	>50
12	TH3-87-2 (TH12)	>50	33	TH4-109-6 (TH33)	>50
13	TH3-103-1K2 (TH13)	>50	34	TH4-121-4 (TH34)	>50
14	TH3-105-1 (TH14)	>50	35	TH4-123-3 (TH35)	>50
15	TH3-105-4 (TH15)	>50	36	TH4-123-6 (TH36)	>50
16	TH3-105-5 (TH16)	>50	37	TH4-123-7K1 (TH37)	>50
17	TH3-107-1K (TH17)	>50	38	TH4-123-8 (TH38)	>50
18	TH3-109-1 (TH18)	>50	39	TH4-127-6 (TH39)	>50
19	TH3-125-2 (TH19)	>50	40	TH4-129-2 (TH40)	>50
20	TH3-125-4 (TH20)	>50	41	TH4-131-2 (TH41)	>50
21	TH3-131-4K (TH21)	>50	42	TH4-131-4 (TH42)	>50

Table 1. Continued

	Cell viability EC_{50} value(μM)			Cell viability EC_{50} value(μM)	
No.	**Code**	**ROT EC_{50}**	**No.**	**Code**	**ROT EC_{50}**
43	TH4-133-11K (TH43)	>50	64	TH5-3-2K3 (TH64)	>50
44	TH4-133-12 (TH44)	>50	65	TH5-3-4K (TH65)	>50
45	TH4-133-13K (TH45)	>50	66	TH5-3-5K (TH66)	>50
46	TH4-139-7K2 (TH46)	>50	67	TH5-3-6K (TH67)	>50
47	TH4-139-8 (TH47)	>50	68	TH5-9-2K1 (TH68)	>50
48	TH4-141-9 (TH48)	>50	69	TH5-11-4 (TH69)	>50
49	TH4-165-3K (TH49)	>50	70	TH5-15-2K3 (TH70)	>50
50	TH4-165-7 (TH50)	>50	71	TH5-15-4K (TH71)	>50
51	TH4-165-8 (TH51)	>50	72	TH5-17-5 (TH72)	>50
52	TH4-173-3 (TH52)	>50	73	JY1-82-3 (JY1)	>50
53	TH4-179-1K (TH53)	>50	74	JY1-91-3 (JY2)	>50
54	TH4-179-4K2 (TH54)	>50	75	JY1-111-1 (JY3)	>50
55	TH4-179-7K2 (TH55)	>50	76	JY1-115-1 (JY4)	>50
56	TH4-195-2K (TH56)	>50	77	JY1-130-4 (JY5)	>50
57	TH4-197-1K2 (TH57)	>50	78	JY1-132-3 (JY6)	>50
58	TH4-197-2K3 (TH58)	>50	79	JY1-62-1 (JY7)	>50
59	TH4-199-1K1 (TH59)	>50	80	JY1-64-4 (JY8)	>50
60	TH4-199-1K2 (TH60)	>50	81	JY1-64-5 (JY9)	>50
61	TH4-199-2K1 (TH61)	>50	82	JY1-64-7 (JY10)	>50
62	TH4-199-2K2 (TH62)	>50	83	JY1-88-13 (JY11)	>50
63	TH5-3-2K1 (TH63)	>50	84	JY1-89-8 (JY12)	>50

Table 1. Continued

	Cell viability EC_{50} value(μM)			Cell viability EC_{50} value(μM)	
No.	**Code**	**ROT EC_{50}**	**No.**	**Code**	**ROT EC_{50}**
85	JY1-164-5 (JY13)	>50	108	JY1-179-8 (JY36)	>50
86	JY1-99-1 (JY14)	>50	109	JY1-179-9 (JY37)	>50
87	JY1-99-2 (JY15)	>50	110	JY1-180-4 (JY38)	>50
88	JY1-101-1 (JY16)	>50	111	JY1-180-7 (JY39)	>50
89	JY1-101-4 (JY17)	>50	112	JY1-180-10 (JY40)	>50
90	JY1-102-4 (JY18)	>50	113	JY1-181-3 (JY41)	>50
91	JY1-104-3 (JY19)	>50	114	JY1-181-4 (JY42)	>50
92	JY1-120-3 (JY20)	>50	115	JY1-181-5 (JY43)	>50
93	JY1-146-1 (JY21)	>50	116	JY1-181-7 (JY44)	>50
94	JY1-153-2 (JY22)	>50	117	JY1-181-8 (JY45)	>50
95	JY1-154-2 (JY23)	>50	118	JY1-182-3 (JY46)	>50
96	JY1-158-2 (JY24)	>50	119	JY2-182-4 (JY47)	>50
97	JY1-162-1 (JY25)	>50	120	JY1-184-9 (JY48)	>50
98	JY1-164-4 (JY26)	>50	121	JY1-188-4 (JY49)	>50
99	**JY1-165-8 (JY27)**	**13.8**	122	JY1-189-1 (JY50)	>50
100	JY1-166-3 (JY28)	>50	123	JY1-190-1 (JY51)	>50
101	JY1-167-6 (JY29)	>50	124	JY1-190-16 (JY52)	>50
102	JY1-168-6 (JY30)	>50	125	JY1-191-3 (JY53)	>50
103	JY1-170-5 (JY31)	>50	126	JY1-193-2 (JY54)	>50
104	JY1-170-6 (JY32)	>50	127	JY1-193-3 (JY55)	>50
105	JY1-172-2 (JY33)	>50	**128**	**JY1-134-4 (JY56)**	**6.5**
106	JY1-173-1 (JY34)	>50	**129**	**Gerontoxanthone C (GXC)**	**5.6**
107	JY1-179-6 (JY35)	>50			

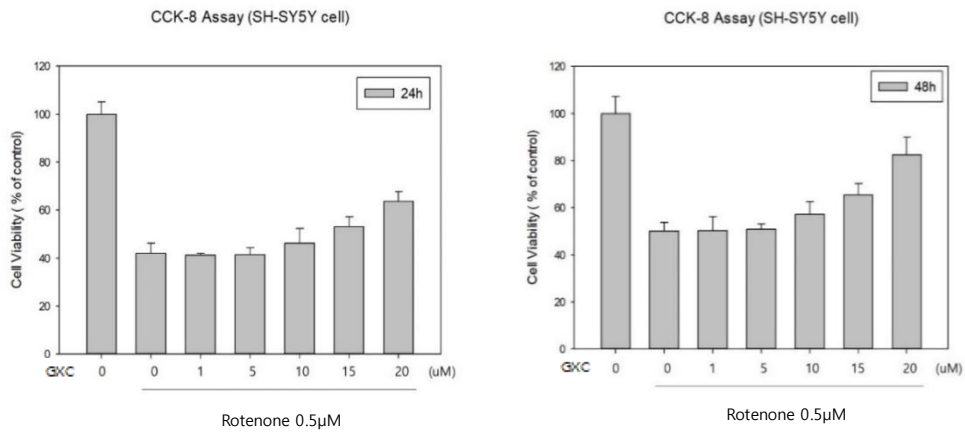

Figure 2. Protective effect of GXC on rotenone-induced cytotoxicity in SH-SY5Y cells

Cells were cultured in 96-well plate for 24 h, and GXC(1-20μM) were simultaneously treated with rotenone (0.5 μM) for 24 h or 48 h. Cell viability were measured by CCK-8 assay. Deta represent the mean ± SD of three independent experiments.

3.2. Protective effect of GXC on rotenone-induced collapse of MMP

The three isolated compounds which protected rotenone-induced cytotoxicity (Table 1) were also evaluated to determine its protective effects on rotenone-induced collapse of MMP. Rotenone induces the rapid collapse of MMP in SH-SY5Y cells. In our results, rotenone-induced collapse of MMP was peaked at 2 hours after treatment rotenone 0.5μM. To evaluate the effect of compounds on MMP, SH-SY5Y cell were pre-treated by GXC for 24h. After then, MMP was disrupted by rotenone. As shown in Table 2, three isolated compounds protected rotenone-induced collapse of MMP. Among three isolated compounds, GXC showed potent neuroprotective effects on rotenone-induced MMP collapse with an EC_{50} values of 6.9 μM. GXC protect rotenone-induced MMP collapse concentration-dependent manner (Fig. 3).

Table 2. Protective effect of isolated compounds from *C.tricuspidata* on rotenone-induced collapse of MMP

	MMP EC_{50} value(μM)	
No.	**Code**	**ROT-induced EC_{50}**
1	JY1-165-8 (JY27)	14.79
2	JY1-134-4 (JY56)	6.85
3	**Gerontoxanthone C (GXC)**	6.9

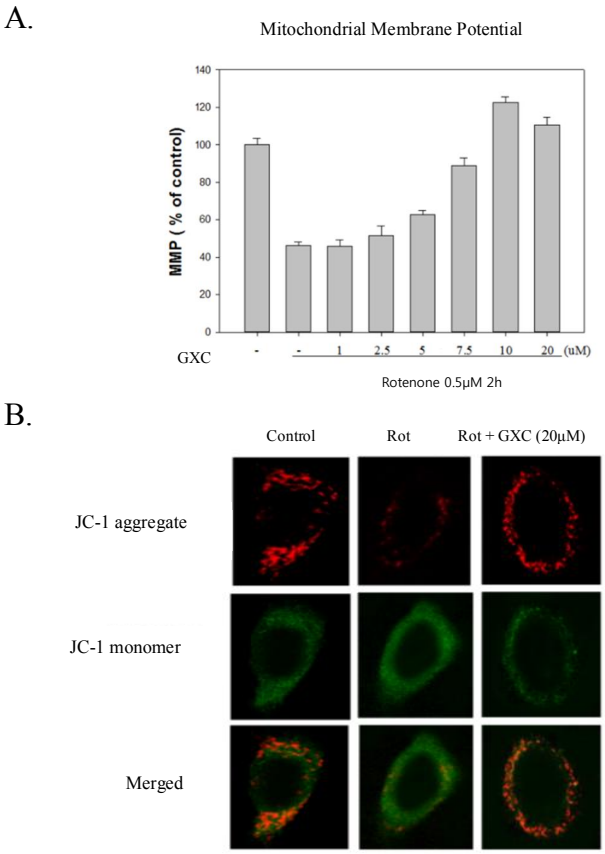

Figure. 3 Protective effect of GXC on rotenone-induced collapse of MMP in SH-SY5Y cells

The cells were pre-treated with different concentrations of GXC (1 - 20 μM) for 24 h and subsequently treated with rotenone (0.5 μM) for another 2 h. The cells were stained with JC-1 dyes for 30 min and determined by FACS analysis using FL-1 and FL-2 channel. The values of fluorescence intensity were obtained from histogram statistic of CellQuest software. (A) The cells

were pre-treated with different concentrations of GXC (20 μM) for 24 h and subsequently treated with rotenone (0.5 μM) for another 2 h. The cells were stained with JC-1 dyes for 30 min and determined by confocal microscopy. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Cellular morphologies were visualized with a fluorescence microscope (×400). Representative images from three independent experiments are shown. (B)

3.3. Inhibition effects of treatment with GXC alone on mTOR activity

mTOR has been known as a key regulator of autophagy. The residual mTORC1 activity may prevent Ulk1 activation by phosphorylation at S757, thus minimizing autophagy initiation. Conditions that inhibit mTORC1, enhanced the activity of ULK1 complex (ULK1-Atg13-FIP200) and triggered autophagy. We evaluated mTOR, ULK-1 protein expression in SH-SY5Y cells. GXC inhibited mTOR protein activity time and concentration dependently. GXC also decreased phosphorylation(S757) of ULK1 time and concentration dependently. (Fig. 4)

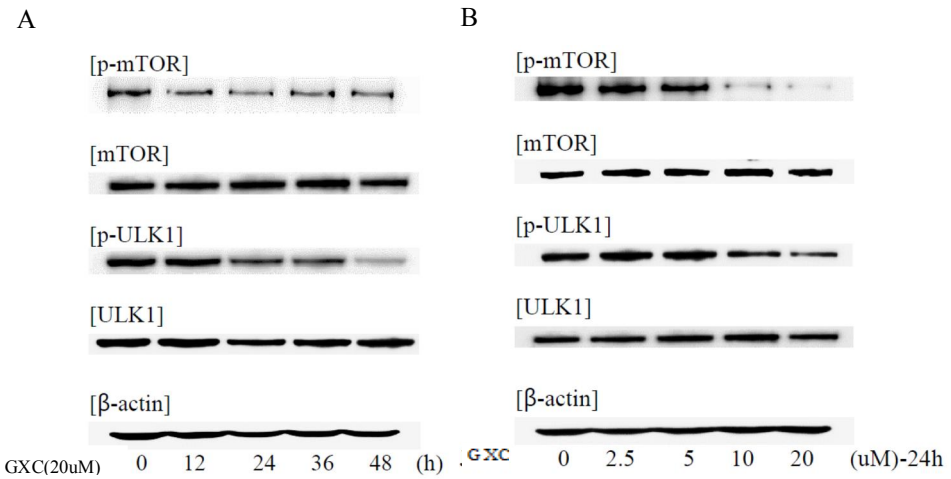

Figure 4. Inhibitory effect of GXC on mTOR activity in SH-SY5Y cells

The cells were treated with GXC for different durations (0 - 48 h, 20 μM) and concentrations (0-20 μM, 24 h). After treatment, the total protein was extracted and determined protein expression level by western blot assay. Representative data from three independent experiments are shown.

3.4. Effects of treatment with GXC alone on intracellular signaling proteins associated with autophagy

Microtubule-associated protein 1A/1B-light chain 3 (LC3) is an autophagosome marker. The conversion of the soluble form (LC3-I) in the cytosol to the autophagosome-bound form (LC3-II) indicates an increase in autophagosome formation, which is typically proportional to the increase in autophagic flux. LC3-binding protein, p62 is also used as a marker to monitor autophagosome. We evaluated LC3, p62 protein expression in SH-SY5Y cells. The expression of LC3 and p62, markers of autophagosome formation, increased after treatment with GXC alone, in a time- and concentration-dependent manner. (Fig. 5)

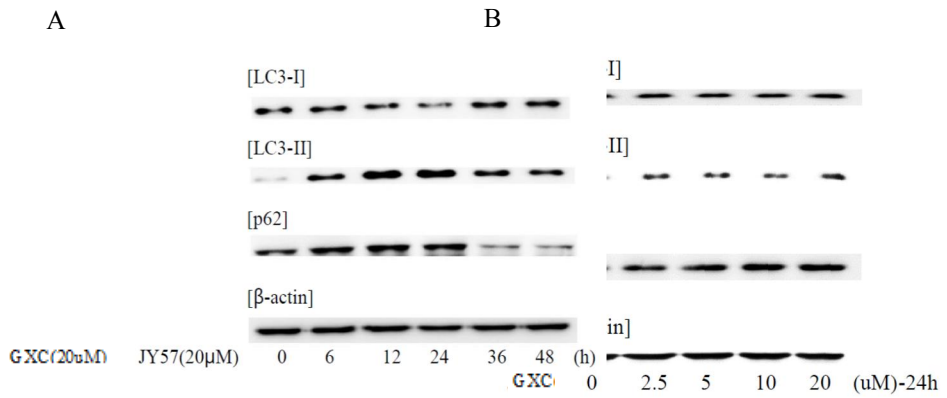

Figure 5. Effect of GXC on LC3, p62 protein expression in SH-SY5Y cells

The cells were treated with GXC for different durations (0 - 48 h, 20 μM) and concentrations (0-20 μM, 24 h). After treatment, the total protein was extracted and determined protein expression level by western blot assay. Representative data from three independent experiments are shown.

3.5. Effects of GXC on levels of intracellular signaling proteins associated with autophagy in rotenone-induced neurotoxicity

Treatment of SH-SY5Y cells with 0.5 μM rotenone alone didn't effect on mTOR expression and ULK1 expression. However, treatment with the combination of rotenone + GXC (2.5, 5, 10 or 20 μM) decreased the activity of mTOR and ULK1, which is an upstream autophagy pathway marker in a concentration-dependent manner (Fig. 6). The levels of LC3-II and the levels of p62, a standard marker of autophagy, increased significantly in a concentration-dependent manner when treated with the combination of rotenone and GXC (Fig. 7). We also used fluorescence microscopy to evaluate accumulation of LC3-II, which implies the formation of autophagosome. The accumulation of LC3-II increased after treatment of GXC (20μM) alone and treatment of GXC (20μM) with rotenone(0.5μM) (Fig. 8).

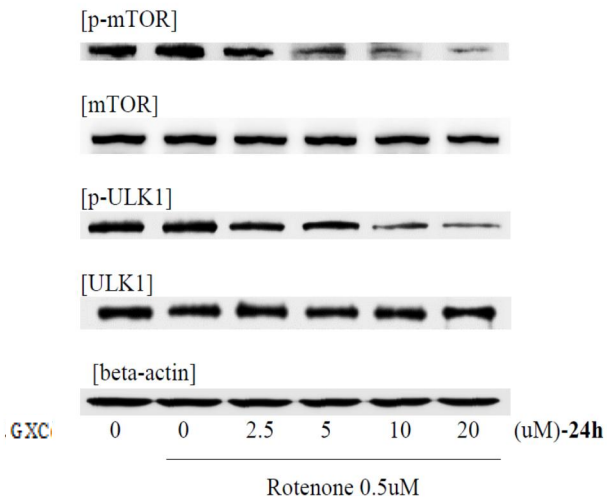

Figure 6. Effects of GXC on mTOR activity in SH-SY5Y cells with rotenone treatment

The cells were treated with GXC for different concentration (0-20 μM) with rotenone (0.5 μM) for 24 h. After treatment, the total protein was extracted and determined protein expression level by western blot assay. Representative data from three independent experiments are shown.

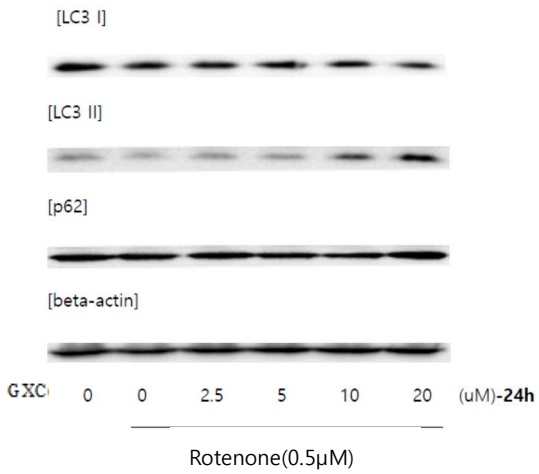

Figure 7. Effects of GXC on LC3 and p62 expression in SH-SY5Y cells with rotenone treatment

The cells were treated with GXC for different concentration (0-20 μM) with rotenone (0.5 μM) for 24 h. After treatment, the total protein was extracted and determined protein expression level by western blot assay. Representative data from three independent experiments are shown.

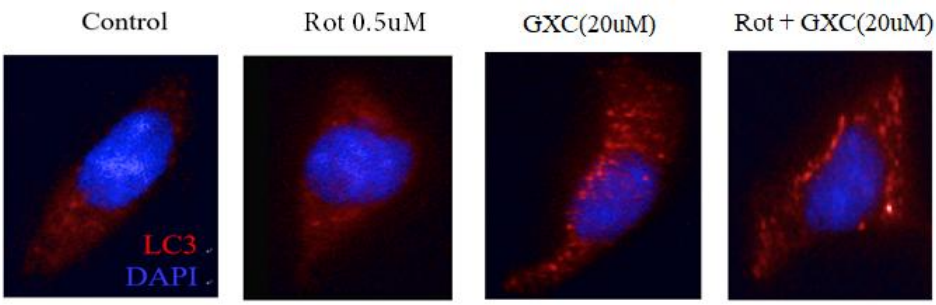

Figure 8. Effects of GXC on autophagosome formation in SH-SY5Y cells with or without rotenone treatment

The cells were treated with rotenone(0.5μM) alone, GXC(20μM) alone, and rotenone(0.5μM)+GXC(20μM) for24h. Cells were fixed and the nuclei were visualized with DAPI staining. LC3 was detected by immunostaining was analyzed and compared with control. Puncta represent autophagosome formation. Cellular morphologies were visualized with a fluorescence microscope (×400). Representative images from three independent experiments are shown.

3.6. GXC prevents rotenone-induced cytotoxicity in an autophagy-dependent manner

Bafilomycin A1 is a known inhibitor of the late phase of autophagy. It prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes. Bafilomycin A1 acts by inhibiting vacuolar H^+- ATPase (V-ATPase). As shown in Fig. 9, GXC (20μM) rescued rotenone-induced cell death and this effect was prevented by bafilomycin A1(20nM).

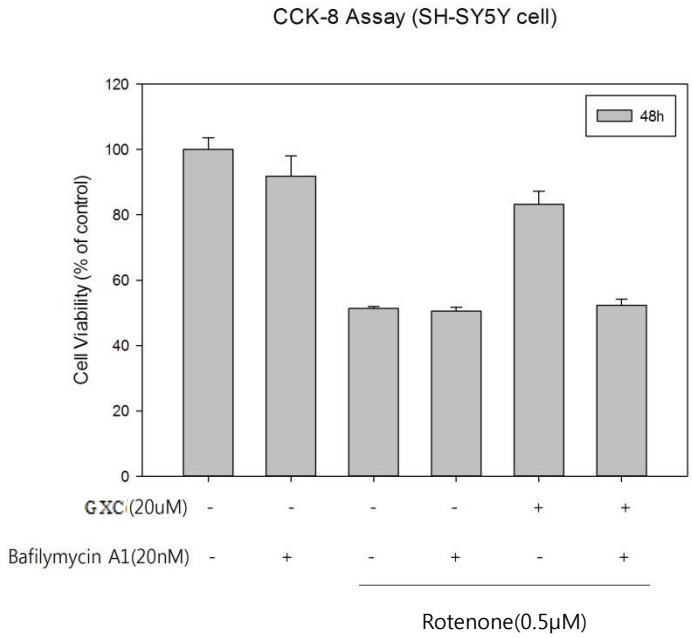

Figure 9. The protective effect of resveratrol against rotenone-induced neurotoxic apoptosis is associated with autophagy

SH-SY5Y cells treated with/without 20 μM GXC for 48 h were treated with/without 0.5 μM rotenone in the presence/absence of 20 nM bafilomycin A1. The cell viability was determined by CCK-8 assay.

4. Discussion

PD is the most common neurodegenerative movement disorder. The primary pathogenesis of PD includes progressive degeneration of dopaminergic neurons in substantia nigra. Furthermore, Lewy bodies, abnormal aggregates of protein that are primarily composed of α-synuclein, have been identified as a distinctive feature of the disorder. Misfolded proteins in PD are degraded via the following two pathways. The ubiquitin-proteasome sys-tem (UPS) selectively degrades short-lived intracellular and plasma membrane proteins under basal metabolic conditions. In contrast, the autophagy-lysosome system (ALS) degrades long-lived, stable proteins, and this is the only pathway that can recycle large organelles such as mitochondria.

In our study, human SH-SY5Y cells exposed to rotenone were used as an in vitro model of PD to explore the effects of GXC on rotenone neurotoxicity and its underlying mechanisms. These findings indicated that GXC could attenuate cell apoptosis and MMP decrease induced by rotenone. Moreover, the protective effects were partially blocked upon treatment with autophagy inhibitors such as bafilomycin A1.

The induction of autophagy by the inhibition of mTOR complex 1

(mTORC1) is conserved from yeast to mammals and acts upstream of the UNC51-like kinase 1 (ULK1) complex. Compared with control group, the expression of p-mTOR and p-ULK1 was decreased by GXC treatment, showing that GXC inhibited mTOR activity. Also, the ratio of LC3-II/I was elevated by GXC, showing that GXC induced autophagy in SH-SY5Y cells.

In the present study, bafilomycin A1 was used to inhibit autophagosome-lysosome fusion, which prevented GXC -induced inhibition of cell death and GXC-induced acidic vesicular organelle formation. This suggests that GXC attenuates neurotoxicity through increased autophagolysosome formation.

Autophagy defects can occur at different stages of the pathway in different diseases, and this may influence treatment strategies. Defects in autophagosome formation may be amenable to drugs that enhance autophagosome biogenesis mTOR inhibitors may therefore be beneficial in this context. In conclusion, we demonstrated the neuroprotective effect of GXC in SH-SY5Y cells against rotenone-induced neurotoxicity and MMP collapse, with inhibition of mTOR activity. The mechanism of neuroprotection is likely to be associated with increased autophagy. The neuroprotective effect of GXC on rotenone-induced dopaminergic

neurotoxicity via modulation of autophagy provides a new therapeutic strategy for the treatment of PD.

5. Conclusion

In conclusion, this study demonstrated that GXC protected against rotenone-induced neuronal cell death and collapse of MMP in SH-SY5Y cells. Our results showed that GXC alone increased ULK1 activity by inhibiting mTOR activity, upstream autophagy pathway, and increased LC3-II and p62, autophagy associated proteins. Also, GXC inhibits mTOR and ULK1 activity, and increased LC3-II and p62 in rotenone-induced neurotoxicity. However, the neuroprotective effect of GXC was inhibited by bafilomycin A1, suggesting their neuroprotective effect is partly due to the induction of autophagy. Based on our results, we suggest that GXC might be promising candidates for the therapy of PD via inducing autophagy.

References

Alexander Panov, S. D., Natalia Shalbuyeva, Georgia Taylor, Todd Sherer, and J. Timothy Greenamyre (2005). "Rotenone Model of Parkinson Disease MULTIPLE BRAIN MITOCHONDRIA DYSFUNCTIONS AFTER SHORT TERM SYSTEMIC ROTENONE INTOXICATION." JOURNAL OF BIOLOGICAL CHEMISTRY **280**(51): 42026–42035.

Angeleen Fleming, T. N., Tamotsu Yoshimori & David C Rubinsztein (2011). "Chemical modulators of autophagy as biological probes and potential therapeutics." Nature Chemical Biology **7**(1): 9-17.

Barbara S. Connolly, M. A. E. L., MD (2014). "Pharmacological Treatment of Parkinson Disease." JAMA **311**(16): 1670-1683.

Beal, M. F. (2001). "EXPERIMENTAL MODELS OF PARKINSON'S DISEASE." Neuroscience **2**: 325-332.

Burton J. Mader, V. N. P., Hilary M. Flippo, Barbara J. Klocke, Kevin A. Roth, Leandra R. Mangieri and John J. Shacka (2012). "Rotenone Inhibits Autophagic Flux Prior to Inducing Cell Death." ACS Chem. Neurosci **3**: 1063−1072.

Chang Hwa Jung, S.-H. R., Jing Cao, Neil Michael Otto, and Do-Hyung Kim (2010). "mTOR regulation of autophagy." FEBS Lett. **584**(7): 1287-1295.

David C. Rubinsztein, P. C., and Beth Levine (2012). "Autophagy modulation as a potential therapeutic target for diverse diseases." Nat Rev Drug Discov. **11**(9): 709–730.

David T.Dexter, P. (2013). "Parkinson disease: from pathology to molecular disease mechanisms." Free RadicalBiologyandMedicine **62**: 132-144.

EJ, K. L. a. W. (2017). "Mitochondrial Dysfunction and Mitophagy in Neurodegenerative Diseases." Cell Dev Biol **6**(2): 1-9.

Eleftherios Karanasios, E. S., Maria Manifava, Takeshi Kaizuka, Noboru Mizushima, Simon A. Walker, Nicholas T. Ktistakis (2013). "Dynamic association of the ULK1 complex with omegasomes during autophagy induction." Journal of Cell Science **126**(22): 5224-5238.

Eve M. Simcox, A. R. a. D. T. (2013). "Monitoring mitochondrial dynamics and complex I dysfunction in neurons: implications for Parkinson's disease." Biochemical Society Transactions **41**(6): 1618–1624.

Guan, Y. C. K. a. K.-L. (2015). "mTOR: a pharmacologic target for autophagy regulation." The Journal of Clinical Investigation **125**(1): 25-32.

Guillermo Mariño, M. N.-S., Eric H. Baehrecke and Guido Kroemer (2014). "Self-consumption: the interplay of autophagy and apoptosis." MOLECULAR CELL BIOLOGY: 1-14.

Hee Ju Kim, J. K., Ki Sung Kang, Keun Taik Lee and Hyun Ok Yang (2014). "Neuroprotective Effect of Chebulagic Acid via Autophagy

Induction in SH-SY5Y Cells." Biomolecules and Therapeutics **22**(4): 275-281.

Jaeyoung Kwon, N. T. H., Dong-Woo Kim, Bang Yeon Hwang, Hak Ju Lee, Woongchon Mar, and Dongho Lee (2014). "Neuroprotective Xanthones from the Root Bark of Cudrania tricuspidata." Nat. Prod **77**: 1893−1901.

Jin H. Son, J. H. S., Kyung-Hee Kim, Ji-Young Ha and Ji Young Han (2012). "Neuronal autophagy and neurodegenerative diseases." EXPERIMENTAL and MOLECULAR MEDICINE **44**(2): 89-98.

Jing Li, S. G. K., and John Blenis (2014). "Rapamycin: one drug, many effects." Cell Metab. **19**(3): 373-379.

Joungmok Kim, M. K., Benoit Viollet and Kun-Liang Guan (2010). "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1." nature cell biology **13**(2): 132-141.

Jr., T. E. H. a. J. C. L. (2003). "TOR Signaling." Science Signaling **212**(15): 1-17.

Jun Li Yang, T. K. Q. H., Basanta Dhodary, Ji Yeon Seo, Hyunju Kim, Junsoo Park, Won Keun Oh (2016). Tetrahedron: 1-10.

Khaled Radad, R. M., Wolf-Dieter Rausch (2015). "Rapamycin protects dopaminergic neurons against rotenoneinduced cell death in primary mesencephalic cell culture." Folia Neuropathol. **53**(3): 250-261.

Klionsk, Z. Y. a. D. J. (2009). "An Overview of the Molecular Mechanism of Autophagy." Curr Top Microbiol Immunol. **335**: 1-32.

Levine, G. K. a. B. (2008). "Autophagic cell death: the story of a misnomer." MOLECULAR CELL BIOLOGY: 1-7.

Levine, Y. L. a. B. (2015). "Autosis and autophagic cell death: the dark side of autophagy." Cell Death and Differentiation **22**: 367-376.

Li Yu, Y. C., and Sharon A. Tooze (2017). "Autophagy pathway: Cellular and molecular mechanisms." Molecular Cell Biology of Autophagy: 1-9.

Lingling Hou, N. X., Ling Liu, Jinsha Huang, Chao Han, Guoxin Zhang, Jie Li, Xiaoyun Xu, Zhicheng Lin and Tao Wang (2015). "Lithium protects dopaminergic cells from rotenone toxicity via autophagy enhancement." BMC Neuroscience **16**(82): 1-10.

Lingling Wu, Z. F., Shaoyuan Cui, Kai Hou, Li Tang, Jianhui Zhou, Guangyan Cai, Yuansheng Xie, Quan Hong, Bo Fu, Xiangmei Chen (2013). "Rapamycin Upregulates Autophagy by Inhibiting the mTOR-ULK1 Pathway, Resulting in Reduced Podocyte Injury." PLOS ONE **8**(5): e63799-e63799.

Meinel J, R. K., Rausch WD, Reichmann H, Gille G (2015). "Cabergoline protects dopaminergic neurons against rotenone-induced cell death in primary mesencephalic cell culture." Folia Neuropathol. **53**(1): 29-40.

Moe Pwint Phyu, J. T. (2014). "Neuroprotective effects of xanthone derivative of Garcinia mangostana against lead-induced acetylcholinesterase dysfunction and cognitive impairment." Food and Chemical Toxicology **70**: 151-156.

N. XIONG, M. J., C. CHEN, J. XIONG, Z. ZHANG, J. HUANG, L. HOU, H. YANG, X. CAO, Z. LIANG, S. SUN, Z. LIN, AND T. WANG (2011). "POTENTIAL AUTOPHAGY ENHANCERS ATTENUATE ROTENONEINDUCED TOXICITY IN SH-SY5Y." Neuroscience **199**: 292-302.

Nian Xiong, J. X., Min Jia, Ling Liu, Xiaowei Zhang, Zhenzhen Chen, Jinsha Huang, Zhentao Zhang, Lingling Hou, Zhijian Luo, Devina Ghoorah, Zhicheng Lin and Tao Wang (2013). "The role of autophagy in Parkinson's disease: otenone-based modeling." Behavioral and Brain Functions **9**(13): 1-12.

Nixon, R. A. (2013). "The role of autophagy in neurodegenerative disease." nature medicine **19**(8): 983-997.

Przedborski, W. D. a. S. (2003). "Parkinson's Disease: Mechanisms and Models." Cell Press **39**: 889-909.

Qian Zhou, C. L., Wen Liu, Hai Zhang, Ruijie Zhang, Jia Liu, Jinfei Zhang, Chong Xu, Lei Liu, Shile Huang and Long Chen (2015). "Rotenone Induction of Hydrogen Peroxide Inhibits mTOR-mediated S6K1 and 4E-BP1/eIF4E Pathways, Leading to Neuronal Apoptosis." TOXICOLOGICAL SCIENCES **143**(1): 81-96.

Ruben K. Dagda, T. D. B. a. E. J. (2013). "How Parkinsonian Toxins Dysregulate the Autophagy Machinery." International Journal of Molecular Sciences **14**: 22163-22189.

Ruoyang Shi, J. W., Ling Zhao, Xin-Min Li, Tian-Ming Gao & Jiming Kong "Excessive Autophagy Contributes to Neuron Death in Cerebral Ischemia." CNS Neuroscience & Therapeutics **18**: 250-260.

Samantha Giordano, M. D., Saranya Ravi, Matthew Redmann, Xiaosen Ouyang1, Victor M Darley Usmar, and Jianhua Zhang (2014).

"Bioenergetic adaptation in response to autophagy regulators during rotenone exposure." J Neurochem **131**(5): 625-633.

Samantha Giordano, M. D., Saranya Ravi, Matthew Redmann, Xiaosen Ouyang, Victor M Darley Usmar, and Jianhua Zhang (2014). "Bioenergetic adaptation in response to autophagy regulators during rotenone exposure." J Neurochem. **131**(5): 625-633.

Sarkar, S. (2013). "Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers." Biochemical Society Transactions **41**(5): 1103–1130.

Sebastian Alers, A. S. L., Sebastian Wesselborg, and Björn Stork (2012). "Role of AMPK-mTOR-Ulk1/2 in the Regulation of Autophagy: Cross Talk, Shortcuts, and Feedbacks." Molecular and Cellular Biology **32**(1): 2-11.

Seo Young Kang, S. Y. K., Hee Ju Kim, Hee-Tae Kim, Hyun Ok Yang, Wooyoung Jang (2017). "Autophagic modulation by rosuvastatin

prevents rotenone-induced neurotoxicity in an in vitro model of Parkinson's disease." Neuroscience Letters **642**(6): 20-26.

Shorafidinkhuja Dadakhujaev, H. S. N., Eun Joo Jung, Joon Yung Cha, Seon Mi Baek, Ji Hye Ha, Deok Ryong Kim (2010). "Autophagy protects the rotenone-induced cell death in α-synuclein overexpressing SH-SY5Y cells." Neuroscience Letters **472**: 47-52.

T. PAN, P. R., Y. WU,a,b W. XIE, J. JANKOVIC AND W. LE (2009). "RAPAMYCIN PROTECTS AGAINST ROTENONE-INDUCED APOPTOSIS THROUGH AUTOPHAGY INDUCTION." Neuroscience **164**: 541-551.

Tavernarakis, N. K. a. N. (2009). "Autophagy and cell death in model organisms." Cell Death and Differentiation **16**: 21-30.

Thorburn, B. E. F. a. A. (2015). "Recent insights into cell death and autophagy." FEBS Journal **282**: 4279–4288.

Thorburn, T. Y. a. A. (2013). "Autophagy and Cell Death." Essays Biochem. **55**: 105-117.

Tsu-Kung Lin, S.-D. C., Yao-Chung Chuang, Hung-Yu Lin, Chi-Ren Huang, Jiin-Haur Chuang, Pei-Wen Wang, Sheng-Teng Huang, Mao-Meng Tia, Jin-Bor Chen and Chia-Wei Liou (2014). "Resveratrol Partially Prevents Rotenone-Induced Neurotoxicity in Dopaminergic SH-SY5Y Cells through Induction of Heme Oxygenase-1 Dependent Autophagy." International Journal of Molecular Sciences **15**: 1625-1646.

Warren E HOCHFELD, S. L., David C RUBINSZTEIN (2013). "Therapeutic induction of autophagy to modulate neurodegenerative disease progression." Acta Pharmacologica Sinica **34**: 600–604.

국문초록

Rotenone으로 유도한 Parkinson's disease model에서 mTOR 억제로 유도된 autophagy에 의한 꾸지뽕나무 유래 유효성분물질의 신경 보호 효과

이 랑 주

약학과 천연물과학 전공

서울대학교 약학대학 대학원

파킨슨병 (PD)은 심한 운동 능력 결핍, 근육의 경직, 운동완만증 및 도파민성 신경 세포의 소실을 특징으로 한다. PD의 병인은 명확하게 밝혀지지 않았다. 그러나 PD 병인의 마지막 공통 경로는 독소의 과도한 축적과 α-synuclein과 같은 단백질의 잘못 접힘을 초래하고, 손상된 단백질을 분해하지 못하면 PD와 관련된 신경 세포의 죽음을 초래할 수 있다. Autophagy는 응집 된 단백질, 손상된 세포 기관 및 세포 내 병원균

을 제거하는 자기 분해 과정이다. 최근 연구에 따르면 PD 환자의 뇌와 PD의 동물 모델에서 autophagy 경로의 조절 장애가 입증되어 PD의 병인에 있어 autophagy의 중추적 역할을 시사하고있다. 일반적인 살충제이자 미토콘드리아 complex I의 억제제인 rotenone은 도파민성 뉴런의 손실 및 PD의 결과적인 측면을 유도한다. 본 연구에서는 SH-SY5Y 신경모세포종 세포에서 rotenone 관련 기전에 대한 천연물의 영향을 조사하여 PD 치료를 위한 새로운 리드 화합물을 발견했다.

Cudrania tricuspidata (뽕나무과)는 한국, 중국 및 일본에 널리 분포하는 아열대 식물이다. *C. tricuspidata*의 열매는 잼, 주스 및 설탕과 함께 발효 된 알코올 음료에 사용되며, 한국에서 식품으로 상업적으로 생산된다. 또한 *C. tricuspidata*의 피층과 뿌리의 껍질은 염증과 종양에 대한 전통 의학으로 사용되어왔다. 최근의 연구에 따르면 *C. tricuspidata*의 추출물은 산화 스트레스에 의해 유발 된 세포 독성으로부터 뉴런을 보호하고 산화 질소 합성 효소 (NOS)에 대한 억제 효과가 있음이 입증되었다. *C. tricuspidata*로부터 분리 된 화합물은 일부 alkaloids, lignins, coumarins, polysaccharides, 그리고 chromones이고, 주로 xanthones and flavones이다. *C. tricuspidata*의 뿌리 껍질에서 유래 한 xanthones의 6-OHDA로 유발된 신경 독성에 대한 보호 효과가 보고되었다.

본 연구에서는 *C. tricuspidata* 추출물의 활성 성분이 autophagy 유도에 미치는 영향을 연구했다. C. tricuspidata의 뿌리 껍질로부터 분리된 Gerontoxanthone C (GXC) 은 SH-SY5Y 세포에서 mTOR 억제를 통해 autophagy를 유발함으로써 rotenone으로 유도된 신경 세포 사멸 및 미토콘드리아 막 전위 (MMP)의 붕괴를 보호했다. 결과를 토대로, GXC는 손상된 세포 기관의 회복을 촉진하여 PD의 치료에 대한 유망한 후보 물질이 될 수 있음을 시사한다.

주요 단어: 파킨슨병, PD, 신경 보호, autophagy, mTOR, *Cudrania tricuspidata*

학번: 2016-29887